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# THE ORGANIC MATRIX FROM OYSTER SHELL AS A REGULATOR OF CALCIFICATION *IN VIVO*

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## ABSTRACT

Exogenously supplied, C-14 labeled organic matrix from oyster shell inhibited spicule formation by embryos of the sea urchin, *Arbacia punctulata*, as measured by simultaneous incorporation of inorganic C-14. Analysis of isolated spicules showed that the matrix reached the site of crystal growth and became incorporated into crystals. Comparison of the amount of matrix in spicules to inhibitory levels for matrix in an *in vitro*, pH-stat crystallization assay showed that the matrix became incorporated into spicules in an amount sufficient to account for the observed *in vivo* inhibition. The matrix did not act as a general metabolic inhibitor, as measured by respirometry, but rather seemed to have only the specific effect on spicule formation. Calculated and measured values of natural levels of matrix in spicules and urchin tests matched reasonably well with experimentally determined levels that regulated the rate of crystallization *in vivo*. Overall, the results support the idea that matrix fulfills a direct, regulatory role in biomineralization.

## INTRODUCTION

The organic matrices from biological minerals are viewed as complex materials which regulate the growth and morphology of the mineral crystalline components (Mann, 1983; Weiner *et al.*, 1983; Greenfield *et al.*, 1984). The capability of matrix to regulate crystal growth *in vivo* may be manifested *in vitro* as the observed matrix induced inhibition of crystal nucleation and growth and change in crystal morphology when various crystallization assays are employed (Wheeler *et al.*, 1981; Sikes and Wheeler, 1983; Wheeler and Sikes, 1984; Wilbur and Bernhardt, 1985). Presumably, in these assays, matrix directly alters crystallization by binding to nascent crystals, as is the case for various synthetic crystal growth inhibitors (Reddy and Nancollas, 1973; Pearce, 1981).

Extrapolation of the *in vitro* assays to biomineral formation has been supported by the finding of Wheeler and Sikes (1984) that $CaCO_3$ incorporation into the spicules of sea urchin embryos could be inhibited when the embryos were incubated with fractions of water-soluble organic matrix extracted from oysters. However, the site of action of the matrix molecules was unknown.

On one hand, it seemed possible that the oyster matrix may have acted at the sites of spicule crystallization. However, the matrix is composed of relatively large polymers that according to prior studies would fall within the range of 20,000 to 180,000 daltons in molecular weight (Crenshaw, 1972; Krampitz *et al.*, 1976; Wheeler *et al.*, 1981; Wheeler and Sikes, 1985; Wheeler *et al.*, 1986). In view of the sea urchin embryo's frequent impermeability to molecules as small as disaccharides (Chizak, 1975), it

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seemed possible that the site of crystallization *in vivo* would be unobtainable to the matrix. That is, the spicule is shielded from the external environment by several cellular and membranous layers which might act as barriers to the movement of matrix from the external medium to the site of crystallization (Okazaki, 1975).

An alternative way that matrix might affect $CaCO_3$ deposition would be by interfering with metabolism in some way that would indirectly result in an inhibition of calcification itself. It is also possible, of course, that the inhibitory effect of oyster shell matrix on sea urchin spiculation results from the combined activity of both the direct and indirect mechanisms of action.

The purpose of this paper is to clarify the mechanisms by which exogenously supplied matrix inhibits spiculation in the sea urchin embryo. The results provide evidence that matrix can inhibit and thus possibly otherwise regulate $CaCO_3$ crystallization *in vivo* by specifically binding to biomineral.

## Materials and Methods

### *Sea urchin embryo culture*

Specimens of *Arbacia punctulata* were collected by divers from the sea wall at the State Park near Panama City, Florida. Collections were made from October through April when these urchins are fertile. On the day of collection, the urchins were transported to the laboratory in an aerated cooler containing natural seawater at 17 to 24°C, then transferred to an 80-gallon aquarium containing artificial seawater (ASW, Aquarium Systems) at 20°C with a salinity matched to that of the natural seawater (specific gravity 1.021 to 1.025). The urchins were fed lettuce every day and could be maintained in a healthy condition in the aquarium for several months.

Cultures of urchin embryos were prepared using standard methods (Sikes *et al.*, 1981). Fertilization typically occurred in greater than 90% of the eggs, with plutei development after 3 days incubation at 20°C.

### *Isolation and radioisotopic labeling of matrix*

The organic matrix from oyster shell was prepared as described elsewhere (Wheeler and Sikes, 1985; Wheeler *et al.*, 1986). Basically, this involved extracting powdered shell in a dialysis bag against 10% EDTA at pH 8.0 until the mineral was dissolved, with the organic matrix retained in the bag. The insoluble component of matrix was removed by centrifugation at 30,000 $\times$ *g* for 20 min. The soluble matrix was dialyzed and concentrated using a Millipore Minitan ultrafiltration system with a nominal molecular weight exclusion limit of 10,000 daltons. Next the matrix was fractionated by gel filtration chromatography on Sephacryl S-300 (Pharmacia). The material that eluted in a broad peak with a median molecular weight of about 20,000 to 40,000 daltons was used in the incorporation studies and is hereafter referred to as "matrix."

The matrix was labeled using the method of Rice and Means (1971) in which C-14 methyl groups were attached to the protein amino groups by reductive alkylation using approximately 2 $\mu$moles of C-14 formaldehyde (ICN Radiochemicals, 40 mCi/mmol) per mg protein. The activity of matrix as determined by use of crystallization assays as well as the chromatographic profile of the matrix were unaffected by the chemical modification. The specific activity of the matrix for the *in vitro* studies was $2.90 \times 10^6$ DPM/mg but was increased for the *in vivo* studies to $6.74 \times 10^6$ DPM/mg protein by increasing the specific activity of the C-14 formaldehyde used during preparation of the labeled protein.

All protein weights were obtained using the Miller (1959) modification of the method of Lowry *et al.* (1951). Matrix was nearly unreactive to the Bradford reagent (Bradford, 1976).

*Radioisotopic measurements*

*In vivo $CaCO_3$ deposition.* Embryos were harvested by hand centrifugation after incubating for two days. By this time, they had reached prism stage when spicules are first becoming visible under polarized light. Embryos were resuspended in fresh, filtered ASW at about 2000 embryos per ml. In some experiments involving uptake of C-14 labeled matrix, the concentration of embryos was raised to 5000 per ml to increase the detectability of the isotope in the samples.

To measure $CaCO_3$ deposition, radioisotope was added at a nominal value of 1 $\mu$Ci/ml of culture from a stock of dissolved inorganic C-14 (DIC-14) in distilled water at 1 mCi/ml (ICN Pharmaceuticals, 53 mCi/mmole). The incubation vessels were 50 ml Erlenmeyer flasks with rubber stoppers covered with parafilm. This suppressed exchange of radioisotopic DIC-14 with the atmosphere. The embryos were incubated in 12 ml at 20 to 22°C on a gyratory shaker table set at 90 rpm to ensure uniform suspension of the embryos in the incubation medium. At intervals during the first 5 hours, 0.5 ml samples of the cultures were collected onto glass fiber filters (Gelman A/E, 25 mm, 0.4 $\mu$m retention) under vacuum at 7 psi. In some experiments, the incubations were allowed to proceed for 24 hours in flasks with cotton stoppers until the embryos began to become inactive as determined by examining swimming and ciliary activity by light microscopy. The embryos in 10 $\mu$l droplets were counted using a binocular microscope, with at least 10 droplets counted per treatment.

Samples on filters were immediately rinsed with 10 ml of unlabeled ASW then placed on a tray in a fume hood to dry overnight. This promoted removal of unincorporated C-14. Next the samples were vortexed in 20 ml scintillation vials containing 10 ml of scintillation cocktail (Beckman MP), then counted using a Beckman 5801 liquid scintillation counting system.

Following initial radioisotopic counting, some samples were treated with 0.3 ml of 1.0 *N* HCl added directly into the scintillation fluid. This lowered the pH of the fluid so that any DIC-14 including that incorporated into $CaCO_3$ in the sample was converted to $CO_2$. The vials were left open in the fume hood and vented for at least 12 hours. Control curves showed that this interval was sufficient to allow greater than 99% of the $^{14}CO_2$ to be exchanged with the atmosphere (Dillaman and Ford, 1982). The samples were then recounted with the residual counts attributed to acid-stable, non-volatile organic components (Sikes *et al.*, 1980).

*Incorporation of C-14 matrix* in vitro. The pH-stat crystallization assay was used as described elsewhere (Wheeler and Sikes, 1984) to measure the effects and the incorporation of matrix during $CaCO_3$ formation *in vitro*. In short, these assays were conducted at 25°C in 25 ml of a vigorously stirred solution containing 500 m*M* NaCl, 10 m*M* KCl, and 10 m*M* DIC with an initial pH of 8.45 to 8.50. To initiate crystal growth, 125 $\mu$l of a 2 *M* $CaCl_2$ solution was added to give a concentration of 10 m*M* Ca. This lowered the pH to approximately 8.3. Following an induction period of 2 to 4 minutes, $CaCO_3$ starts to form. This results in a decline in the pH of the solution according to the overall reaction, $Ca^{2+} + HCO_3^- = CaCO_3 + H^+$. However, the pH of the crystal growth solution was held constant at pH 8.30 ± 0.02 by autotitration with microliter quantities of 0.5 *N* NaOH using a Metrohm pH-stat system (Model 655 Dosimat with a 1.0 ml piston burette and a Model 614 Impulsomat) attached to a pH meter (Beckman model 3500). The progress of crystal growth was then followed

by the quantity of titrant added, which for the concentrations involved was essentially equivalent to the quantity of $CaCO_3$ formed.

C-14 matrix was added during experiments after crystals had formed in an amount equivalent to 25 $\mu$moles of added titrant. The percent inhibition was determined by comparing the rates of crystal growth before and after the addition of matrix.

The incorporation of matrix into crystals was determined by periodically removing 1 ml aliquots from the growth medium and collecting the crystals onto cellulose triacetate filters (Gelman type GA-8, 25 mm diameter, 0.2 $\mu$m pore size). The crystals were dissolved from the surface of the filters by washing them twice with 2 ml of 0.1 *N* HCl. The entire 4 ml was added to 10 ml of Beckman EP scintillation fluid and counted. Corrections for adsorption of isotope to filters were not significant and were made by use of control filters without crystals.

*Incorporation of C-14 matrix into spicules.* After incubation of embryos in the presence of C-14 matrix, it was necessary to isolate the spicules from the embryos so that any matrix that may have become associated with the spicules could be detected. To do so unambiguously, the isolated spicules had to be absolutely free from attached cellular debris. Accordingly, we needed to develop a technique for obtaining clean spicules.

Following incubations in which C-14 matrix was added to the culture, embryos were harvested in the normal way. Then they were resuspended in a solution of 5.25% sodium hypochlorite in a test tube that was placed in a sonicator (14 watts, Electromation Components) for 15 minutes. Following this treatment, spicules could be separated from cellular remains by hand centrifugation. This procedure was repeated two more times, with the spicules finally resuspended in isopropyl alcohol. Visual examination at 450× revealed complete, well-formed spicules with no trace of cellular debris.

This treatment was selected after a number of milder methods had failed. For example, sonication in isopropyl alcohol, 1% and 5% Triton, 1% and 5% sodium dodecyl sulfate, 1% and 5% dimethyl sulfoxide all yielded well-formed spicules upon hand centrifugation to separate spicules from cellular debris. However, in every case, a fairly extensive amount of cellular debris remained, particularly in the more fenestrated portions of spicules. This problem was compounded by the fact that spicules grown in the presence of matrix were even more prone to retain cellular attachments following these treatments with mild solvents. On the other hand, treatment with 1 *N* NaOH not only removed the cellular debris but also rapidly led to disintegration of the spicules themselves.

Other workers had shown previously that $CaCO_3$ structures treated with strong oxidants would retain an internal organic matrix (Crenshaw, 1972; deJong *et al.*, 1976). Similarly, hypochlorite has been used by others to prepare clean spicules from sea urchins (Okazaki, 1975; Mintz *et al.*, 1981) without apparent loss of matrix (Benson *et al.*, 1986).

Following hypochlorite treatment, the spicules were washed 3 times in and then resuspended in 5 ml of $10^{-4}$ *M* NaOH. Dilute base was used for this purpose because washing in distilled water led to partial dissolution of the spicules. Next, aliquots of the spicule preparation were taken for assessment of radioactivity by liquid scintillation.

### *Respirometry*

The method for measurement of $O_2$ consumption by the sea urchin embryos has been described elsewhere (Sikes *et al.*, 1981). The method involves the use of an $O_2$ electrode and a Plexiglas chamber designed for assessment of respiration by cellular

suspensions in 3 ml. The electrode (Beckman, Ag/Au) was energized by an adaptor constructed according to the design of Estabrook (1967) and connected to a voltmeter which was read to the nearest 0.1 mV and recorded by strip chart. Calibration curves were prepared by removing all $O_2$ from saturated solution by addition of $Na_2SO_3$. Saturation values for $O_2$ in seawater were taken from Kester (1975). In addition, filtered ASW to be used in respirometry was allowed to equilibrate with the atmosphere for 1 hour with smooth magnetic stirring prior to use. Confirmation of the saturation values of $O_2$ in seawater was made by use of the Winkler titration (American Public Health Service, 1981) with good agreement with the values of Kester. The respirometer was stable ±1 to 2 mV per hour and full deflection from saturated to anoxic conditions was set at about 50 mV. Embryos were capable of completely depleting media of $O_2$.

### *Statistics*

Linear regression analysis was performed according to Kleinbaum and Kupper (1978) including tests for parallelism and coincidence. Analysis of variance was used to determine significance of differences in the tabular results (Keppel, 1973).

## Results

Matrix inhibited spicule formation in *A. punctulata* embryos (Fig. 1). The incubation medium for this experiment included both DIC-14 and C-14 labeled matrix. This allowed for both the formation of spicules and the incorporation of matrix to be measured in the same experiments. In Figure 1, each point is based on three replicate samples per experiment with a total of three complete experiments (n = 9).

C-14 uptake was significantly suppressed in the presence of matrix ($P < 0.01$). However, the incorporation of acid-stable C-14 in the presence of matrix was significantly elevated relative to control values ($P < 0.01$), presumably due to incorporation

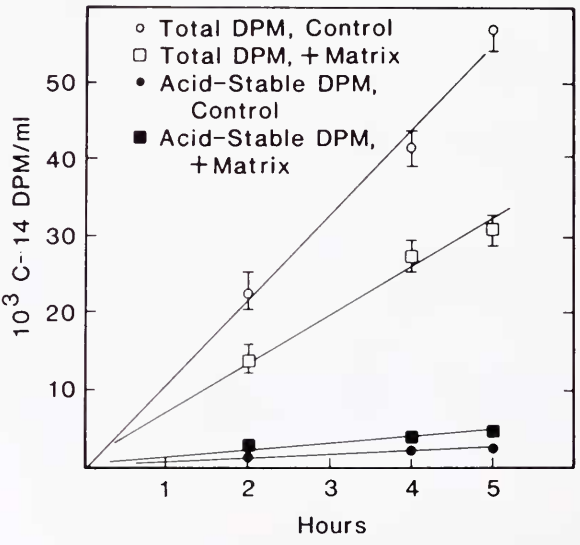


FIGURE 1. Incorporation of dissolved inorganic C-14 and C-14 labeled matrix by embryos of the sea urchin, *Arbacia punctulata*. There were 2800 ± 333 embryos/ml of medium (n = 10). The conditions for these experiments were the same as those listed in Table I.

of the C-14 labeled matrix into the embryos. Using the data of Figure 1, the amount of inorganic C-14 incorporation into spicules was determined as the difference between the total uptake and the acid-stable uptake. By computing this difference based on the slopes of the respective curves, a 48.5% inhibition of spiculation was observed over the first 5 hours of the incubation.

Final measurements were made after 24 hours (Table I). By this time, the urchins had reached the pluteus larva stage but did not develop further due to lack of food. By the end of the incubations, the relative contribution of inorganic C-14 to the acid-stable component became larger. This presumably occurred because the embryos approached saturation with the C-14 labeled matrix while the fixation of inorganic C-14 into organic material was more or less continuous. In any case, the ratio of C-14 in the acid-stable component compared to total DPM values was still higher in the experimental treatment.

Uptake of C-14 labeled matrix alone by urchin embryos was also measured (Table II) in three experiments. In these experiments a treatment at 2°C was used that has been shown to stop respiration and spiculation, but allows the embryos to resume normal activity upon warming (Sikes *et al.*, 1981). There was a temperature-dependent incorporation of C-14 labeled matrix by embryos during the 24 hour incubation ($P < 0.01$). Note also that a small but significant ($P < 0.01$) amount of the temperature-dependent incorporated radioactivity was associated with the spicules that were isolated by the hypochlorite method. In both cases, the temperature-dependent uptake suggested that the incorporation was dependent on metabolism.

*In vitro* crystallization also was suppressed in the presence of C-14 matrix (Fig. 2). An apparent decline in DPM/ml as the experiment progressed was due both to dilution by titrant and to settling of larger crystals even with vigorous stirring. Incorporation of matrix by crystals was corrected for the decline, which did not exceed 10% of initial values for DPM/ml. The concentration dependence of C-14 matrix on $CaCO_3$ crystallization is shown as redrawn chart recordings in Figure 3A. These results were replotted in Figure 3B to give the amount of matrix incorporated per $\mu$mole of NaOH titrated. The plots in Figures 2 and 3 are representative examples from families of curves which were replicated up to 10 times. Standard deviations in the amount of inhibition and the amount of matrix incorporated into crystals at particular levels of matrix were less than 10% of the values reported for these data.

Although doses up to 100 $\mu$g matrix/ml were supplied, there was no significant effect of the matrix on respiration by the embryos (Fig. 4). However, doses this high were essentially completely inhibitory to spicule formation. In these assays, the number

TABLE I

*C-14 incorporation by embryos of the sea urchin,* Arbacia punctulata, *that were incubated for 24 hours in medium containing both DIC-14 and C-14 labeled matrix*

| | Total DPM $\times 10^{-3}$ (means ± SD) | Acid stable DPM $\times 10^{-3}$ (means ± SD) | $\mu$mole $CaCO_3$* per $10^4$ embryos |
|---|---|---|---|
| Control embryos | 765 ± 84.8 | 162 ± 32.4 | 0.766 |
| Experimental embryos | 499 ± 20.4 | 131 ± 93.5 | 0.467 |

* (2.2 $\mu$moles DIC/ml) (total − acid-stable DPM/embryos) (DPM/ml) (2.72**).

** A correction factor to account for simultaneous incorporation of unlabeled respiratory $CO_2$ and DIC-14 by embryos (Sikes *et al.*, 1981).

There were 33,600 ± 4000 embryos (n = 10) in a total of 12 ml of medium. Radioactivity of the medium was $1.48 \times 10^6$ DPM/ml of which $6.93 \times 10^4$ DPM/ml were attributed to C-14 labeled matrix.

TABLE II

*Incorporation of C-14 labeled matrix into whole embryos and isolated spicules of the sea urchin,* Arbacia punctulata

| | Control 2°C (means ± SD) | Experimental 22°C (means ± SD) | μg matrix/ μmole $CaCO_3$* |
|---|---|---|---|
| C-14 matrix in whole embryos, DPM/$10^4$ embryos | 3190 ± 107 | 8232 ± 747 | 11.0 |
| C-14 matrix in isolated spicules, DPM/$10^4$ embryos | 21.5 ± 0.953 | 113 ± 4.47 | 0.20 |

* (Expt'l − Control DPM/$10^4$ embs) (μg matrix/DPM) ($10^4$ embs/0.467 μmole $CaCO_3$).

There were 60.5 μg matrix/ml of medium giving a radioactivity of 59,600 ± 1110 DPM/min (n = 12). The culture contained 5020 ± 599 embryos/ml (n = 10) in 12 ml of total volume.

of embryos/ml was raised to 11,400 ± 1230 (n = 18) to allow a rapid measurement of $O_2$ consumption. This did not seem to affect the embryos adversely, in that the overall amount of $O_2$ consumption measured was 12.3 ± 0.569 nmoles $O_2$/1000 embryos/h (n = 9). This falls into the range of values of respiration by urchin embryos reported by other workers (Yanagisawa, 1975). The value for $O_2$ consumption was computed based on the slope of the curves over the first 15 minutes of an assay. As seen in Figure 4, after this time, the rate of $O_2$ consumption began to decrease, possibly due to damage to the embryos caused by the stirring needed to optimize the response of the oxygen electrode.

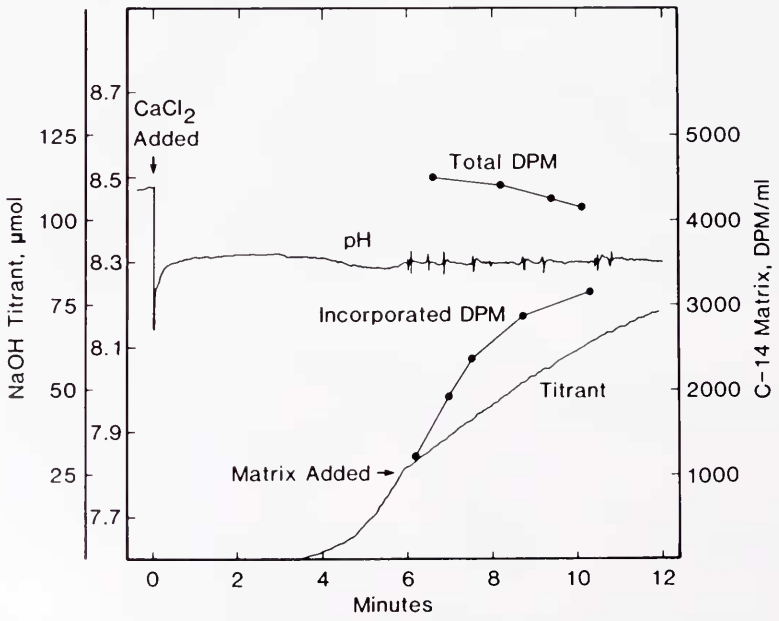


FIGURE 2 The incorporation of C-14 labeled matrix during $CaCO_3$ formation *in vitro* in the pH-stat crystallization assay. C-14 labeled matrix was added at 1.53 μg/ml (4.45 × $10^3$ DPM/ml) after 25 μmoles of NaOH titrant had been delivered by autotitration.

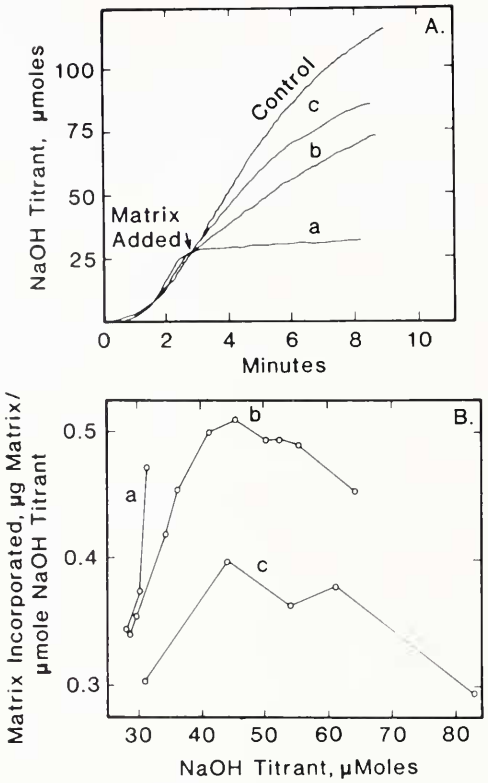


FIGURE 3. The effect of C-14 labeled matrix on $CaCO_3$ crystallization *in vitro* measured using the pH-stat assay. In part A, the control curve represents crystallization in the absence of matrix. Curve *a* shows a 97% inhibition of crystal growth when 1.84 µg matrix/ml was added; curve *b*, 53% inhibition at 1.53 µg matrix/ml; curve *c*, 23% inhibition at 1.22 µg matrix/ml. In part B, the amount of C-14 labeled matrix incorporated into the crystals grown during the experiments of part A is shown.

## DISCUSSION

It is clear that the matrix from oyster shell, when added to the external medium, can reach the site of $CaCO_3$ crystallization during spicule formation by sea urchin embryos. This was evident from the experiments in which C-14 labeled matrix was detected in carefully isolated spicules from urchin embryos that had been grown in the presence of the labeled matrix (Table II). Spiculation was shown to be markedly suppressed in urchins treated with this amount of matrix (Fig. 1), indicating that the matrix did influence $CaCO_3$ deposition. In addition, the residual radioactivity of the spicules that we attributed to the presence of matrix was entirely acid-stable which eliminated the possibility that the C-14 may have been in the form of $Ca^{14}CO_3$, having become available as a source of inorganic carbon as respiratory $CO_2$ (Sikes *et al.*, 1981). These results also show that oyster shell matrix is relatively mobile in the sea urchin embryos despite the numerous intervening cellular and extracellular layers (Okazaki, 1975; Kingsley *et al.*, 1984).

To determine if the oyster matrix associated with spicules was sufficient to account for the observed inhibition of spiculation, the results *in vivo* can be compared to those of the pH-stat, *in vitro* experiments. This comparison seems reasonable because the

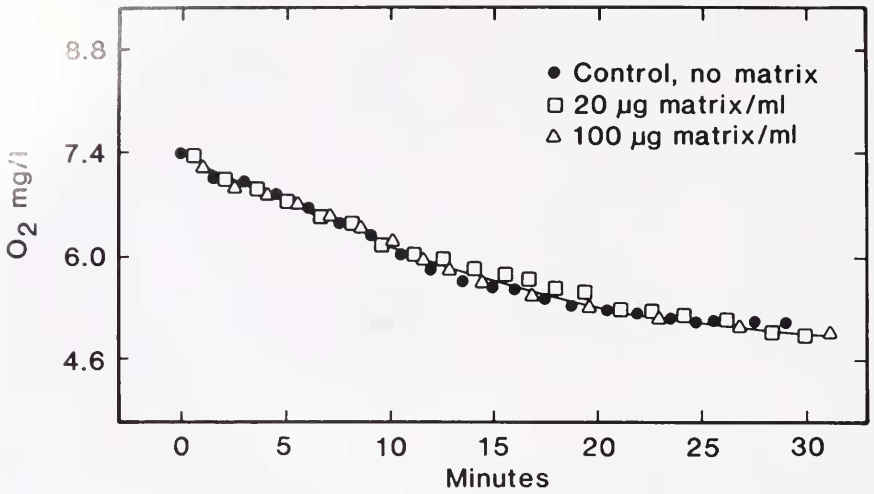


FIGURE 4. The effect of matrix on oxygen consumption by embryos of the sea urchin, *Arbacia punctulata*.

rates of crystal formation *in vitro* and *in vivo* can be similar. That is, based on the results of the radioisotopic incorporation (Fig. 1), urchins deposited 0.0166 μmoles of DIC-14 per ml (2800 embryos) per hour, or after correction for simultaneous incorporation of carbonate that originates as respiratory $CO_2$ (Table I), 0.0452 μmoles of $CaCO_3$ deposition per 2800 embryos per hour. By computing a spherical volume for the embryos using 50 μm as a typical radius based on microscopic examination using an ocular micrometer, each embryo would have a volume of $5.24 \times 10^{-7}$ $cm^3$. Using this figure, we arrive at a value of 30.8 μmoles of $CaCO_3$ deposited per hour per $cm^3$ of embryos. For comparison, the rate of deposition in the pH-stat *in vitro* assay can be read from Figure 2 as 48 μmoles of $CaCO_3$ deposited per hour per $cm^3$ of solution.

Because of the similar rates of crystal formation, it seems reasonable to compare the concentrations of matrix required for inhibition *in vitro* to those that might occur *in vivo*. In the present study, the level of matrix incorporated into mineral that resulted in inhibition *in vitro* (0.4–0.5 μg per μmole $CaCO_3$) was somewhat higher than recorded *in vivo* (0.2 μg per μmole $CaCO_3$) which was consistent with the somewhat higher rate of deposition measured *in vitro* as compared to that *in vivo*. Further, it is important to bear in mind that because of simultaneous secretion of unlabeled matrix by embryos, the value of matrix measured by C-14 incorporation into spicules may be an underestimate of the total level of regulatory matrix to which growing crystals were exposed. Therefore, it can be tentatively concluded that the exogenously supplied C-14 matrix was associated with mineral in quantities sufficient to account for the observed inhibition of *in vivo* mineralization. In addition, because the matrix had no effect on respiration of the embryos, it seems unlikely that there was any indirect inhibitory effect of matrix on spicule formation.

At this point, the possibility that naturally occurring levels of spicular matrix may have regulatory effects similar to those seen with the C-14 matrix should be considered. Although oyster shell was the source of the matrix in the present study, spicular matrix also can inhibit spicule formation by urchin embryos (Swift *et al.*, 1986) and seems

to be similar to oyster shell matrix in composition (Weiner *et al.*, 1983; Benson *et al.*, 1986). Thus it seems likely that the two matrices would elicit similar effects.

The value of 0.2 μg matrix incorporated per 100 μg (μmole) $CaCO_3$ measured during inhibition of spiculogenesis in the present study corresponds with the range of matrix (0.06–0.3% by weight) obtained for sea urchin tests (Pilkington, 1969; Weiner *et al.*, 1983; Swift *et al.*, 1986) and for spicules (approximately 0.1%) isolated in a manner similar to that of the present study (Benson *et al.*, 1986).

These values may include matrix which is not involved in regulatory functions and thus represent estimates for a maximum percent of regulatory matrix in mineral. For example, only 25% of urchin test matrix is a soluble fraction and thus similar to the C-14 matrix used in this study. If we accept that only the soluble fractions can regulate mineral formation; that is, as has been suggested, the insoluble fraction has a structural rather than regulatory role (Weiner, 1984), then the naturally occurring levels of soluble matrix might produce much less dramatic regulatory effects, especially with respect to controlling rate of crystallization. Of course, this has to be true at times during biomineralization, otherwise crystal growth might be terminated altogether.

In reality, the fraction or total proportion of matrix which can regulate growth is unknown. It may be that in some systems most matrix has regulatory capacity at some point between the time it is secreted and its final incorporation in mineral. Further, it is likely that the matrix is deployed *in vivo* such that different portions of the spicules or test receive relatively higher amounts of matrix at different times. This might occur if the organism was slowing or stopping the growth of crystals in one region while promoting crystallization in another.

Overall, this study provides experimental evidence that matrix levels in the approximate range of that found in biomineral can control rate of crystallization. Several authors have pointed out that biomineral growth must be limited, at least periodically (Watabe, 1965; Bevelander and Nakahara, 1969; Crenshaw and Ristedt, 1975; Weiner and Hood, 1975; Wheeler *et al.*, 1981; Borman *et al.*, 1982; Wheeler and Sikes, 1984). Certainly this also is true and it may be that one of the functions of some components of matrix is to stop the formation of a biomineral structure when needed. It is also possible that the inhibition of crystallization observed *in vitro* is not so much a reflection of matrix acting only to stop crystal growth but also to arrange crystal morphology. That is, soluble matrix as well as other inhibitors of crystallization can alter crystal morphology *in vitro* (Nancollas and Reddy, 1974; Kitano *et al.*, 1978; Rohm and Haas, 1983; Wheeler and Sikes, 1984). Presumably this occurs in response to the binding of the inhibitor to an existing crystal growth site with the result that new growth sites develop (Mann, 1983), changing the morphology of the crystal and ultimately resulting in biominerals of many varied forms.

The findings described herein do not contradict prior findings that matrix also may serve sometimes to nucleate or initiate crystal growth (Crenshaw and Ristedt, 1975; Sikes and Wheeler, 1982; Dillaman and Roer, 1984; Greenfield *et al.*, 1984; Wilbur and Manyak, 1984; Addadi and Weiner, 1985; Bernhardt *et al.*, 1986). Many authors have recognized the possibility that matrix may serve at least a dual function in regulation of biomineralization. Exactly how one material, the matrix, can fulfill the various functions assigned to it remains an interesting enigma (Wilbur, 1985).

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